Biochem. J. (1980) **187**, 767–774
Printed in Great Britain

Preparation and Characterization of Chemically Defined Oligomers of Rabbit Immunoglobulin G Molecules for the Complement Binding Studies

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(*Received 7 September 1979*)

Pure dimers, trimers, tetramers and pentamers of rabbit non-immune IgG (immunoglobulin G) or antibody IgG were prepared by polymerization in the presence of the bifunctional cross-linking reagent dithiobis(succinimidylpropionate). Oligomerization was performed either in the presence of polysaccharide antigen and specific monomeric antibody (method A) or by random cross-linking of non-immune rabbit IgG in the absence of antigen (method B). By repeated gel-filtration chromatography, samples prepared by both methods exhibited a single band in analytical sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The electrophoretic mobilities of samples prepared by method A were slightly greater than those for the corresponding samples prepared by method B. This might suggest a role played by antigen in the orientation of IgG molecules within the clusters, which may be more compact than those formed by random cross-linking. The average numbers of cross-linker molecules per oligomer varied between 3 and 6 for clusters made by method A and between 1 and 3 for clusters made by method B. Ultracentrifugal analyses of the oligomers yielded sedimentation coefficients ($s_{20,w}$) of 9.6S for the dimer, 11.2S for the trimer, 13.6S for the tetramer and 16.1S for the pentamer. Comparison of the observed sedimentation coefficients with those predicted by various hydrodynamic models suggested these oligomers possessed open and linear structures. Reduction of the cross-linking molecules converted oligomers into monomeric species of IgG. C.d. spectra of some oligomers studied in the range 200–250nm were essentially the same as that of monomeric IgG molecules, thus strongly suggesting no major conformation changes in IgG molecules within clusters. These oligomers were found to be stable for up to 2 months when stored at −70°C.

The antibody molecules can be viewed as a biochemical transducer that connects the activity of antigen recognition with certain effector functions (e.g. the triggering of complement activation, the release of histamine mediated by mast cells and the induction of lymphoid cells to differentiation) that can be evoked on specific combination with antigen (Metzger, 1974, 1978). The antigen-recognition function is carried in the *N*-terminal region of the IgG molecules (Fab portion), and the effector functions are manifested by the *C*-terminal domains (Fc portion) (Nisonoff *et al.*, 1975). These two functional regions are joined via two segments of the heavy polypeptide chain, designated as the hinge peptides. The appearance of effector functions subsequent to antigen binding has been rationalized by several hypothetical mechanisms (Metzger, 1974, 1978). Briefly, the antigen evokes these new functions either by inducing a conformation change in the distally located Fc region (allosteric model) or by spatially organizing immunoglobulin domains to produce multivalent complexes (associative model). In the latter case the mere polymerization of antibody by multivalent antigen is the necessary and sufficient role played by the antigen. Generally, univalent antigenic determinants are not effective in eliciting the various effector responses mediated by the Fc portion of the molecule (Metzger, 1974, 1978; see, however, Goers *et al.*, 1977). Recent evidence points to aggregation of antibody rather

Abbreviation used: IgG, immunoglobulin G.

0306-3275/80/060767-08$01.50/1

than to the appearance of an altered, antigen-induced, conformational state in the Fc part of IgG as the most likely explanation for the appearance of these new effector functions (Jaton *et al.*, 1976; Wright *et al.*, 1977; Willan *et al.*, 1977*a*,*b*; Dudich *et al.*, 1978; Wright *et al.*, 1978*a*,*b*; Wright, 1978).

Previous studies have made use of immunoglobulin aggregates produced by reaction with antigen, by non-specific cross-linking, by heat aggregation or by cross-linking via bivalent haptens. The drawbacks of these approaches are twofold: the IgG oligomers are undefined and/or unstable (Hyslop *et al.*, 1970). Complement fixation has been observed with clusters containing a minimum of four or five immunoglobulin molecules (Hyslop *et al.*, 1970; Jaton *et al.*, 1976). The production of dimers and trimers of IgG covalently cross-linked via their variable domains by means of a bivalent affinity label has been described (Segal & Hurwitz, 1976; Segal *et al.*, 1979). Dimers and trimers of these complexes exhibit no complement-fixation ability. In the present paper the production, purification and characterization of a series of chemically cross-linked rabbit IgG oligomers ranging in size from dimers to pentamers is reported. These oligomers are stable, yet they can be readily dissociated because of the use of the thiol-sensitive cross-linking agent dithiobis(succinimidylpropionic acid). The IgG oligomers were used in subsequent work for studies of complement fixation (see the accompanying paper, Wright *et al.*, 1980).

Materials and Methods

Synthesis of oligomers

The rabbit anti-(SIII pneumococcal polysaccharide) antibody 45–394 was purified by affinity chromatography as described in detail elsewhere (Jaton *et al.*, 1976). Non-immune rabbit IgG was isolated from sera by $(NH_4)_2SO_4$ and Na_2SO_4 precipitations and by chromatography on DEAE-Sephadex A-50 and Sepharose 6B-CL (Pharmacia Fine Chemicals, Uppsala, Sweden). A 28-sugar hapten was isolated from an acid hydrolysate of the SIII polysaccharide (Jaton *et al.*, 1976). After tyramination (Eichman & Kindt, 1971), this derivative was iodinated with ^{131}I by the procedure of Eichman (1972). The final specific radioactivity was 1120c.p.m./nmol. Oligomers of rabbit IgG were prepared (*a*) by the chemical cross-linking of specific antibody in hapten–antibody complexes and (*b*) by the random chemical cross-linking of non-immune immunoglobulin in concentrated solutions.

Method A

A solution of antibody 45–394 (11.3μM) in 0.1M-sodium borate buffer, pH9.0, was cooled in ice. A 3.5–4mg/ml solution of dithiobis-(succinimidylpropionate) (Pierce Chemicals, Rockford, IL, U.S.A.) in dimethylformamide was likewise cooled in ice. This solution was added dropwise to the solution of antibody with gentle mixing and cooling between additions, 40μl of the cross-linker being used per ml of antibody solution. Immediately thereafter, radioiodinated hapten in 0.1M-sodium borate buffer, pH9.0, at 0°C was added dropwise until the solution became turbid (hapten/binding-site molar ratio approx. 1.8:1). The reaction mixture was kept overnight at 2–4°C, during which time a small immune precipitate was formed. The suspension was dialysed at 4°C against 0.2M-sodium phosphate buffer, pH3.5, containing 1M-NaCl for 24h. After that time the immune precipitate dissolved, and dialysis was continued until the hapten was dissociated and dialysed away from the complexes. The final antibody/hapten molar ratio exceeded 200:1, as determined from measurements of the retained radioactivity. The antibody solution was subsequently dialysed, first against 0.2M-sodium phosphate buffer, pH5.0, overnight, and then against 0.2M-sodium phosphate buffer, pH7.4, for 10h. The antibody was precipitated by the addition of $(NH_4)_2SO_4$ to 50% saturation (at pH7.4). This sample was processed further as described below for method B.

Method B

Purified non-immune rabbit IgG from single sera was concentrated by the addition of an equal volume of saturated Na_2SO_4 at 30°C, the solution was centrifuged at 30°C after 2h, and the precipitate was dissolved in 0.1M-sodium borate buffer, pH9.0. This solution was dialysed overnight against the same buffer at 4°C. Rabbit IgG (200–270μM) was cross-linked by the dropwise addition of 40μl of a 4mg/ml solution of dithiobis(succinimidylpropionate) per ml of antibody in solution. Both solutions had been precooled and were kept in ice. The coupling reaction was allowed to proceed for 2h at 4°C after completion of the addition of the cross-linking reagent. IgG oligomers prepared by either method A or method B were then fractionated from the bulk of non-cross-linked material by passage of up to 3ml samples through tandem columns of Sephadex G-200 (2.6cm × 95cm) and Sephadex 6B-CL (3cm × 95cm) (Pharmacia Fine Chemicals) equilibrated with 150mM-NaCl/10mM-Tris/HCl buffer, pH7.4. Effluent fractions containing predominantly one species as judged by gel electrophoresis (see below) were collected and concentrated by the addition of $(NH_4)_2SO_4$ (0.2g/ml). Redissolved samples at final volumes of 0.5–2ml were resubjected to chromatography on the Sepharose column once or twice until pure fractions of oligomers were obtained. This column was developed in the upward-flow mode with a flow rate

of 16ml/h. After the loading of the protein samples 10ml of buffer containing 10% saccharose was applied to improve the resolution. Pure fractions of oligomers obtained in this manner were stored in the developing buffer at −70°C until used.

Gel electrophoresis

Preparations of IgG oligomers could be conveniently analysed on 14cm slab gels consisting of 7.5% acrylamide (acrylamide/*NN'*-methylenebisacrylamide ratio 75:2, w/w) for monomers to trimers, 5% acrylamide (acrylamide/*NN'*-methylenebisacrylamide ratio 50:1, w/w) for higher oligomers, or a 3–7.5% polyacrylamide-gel gradient (acrylamide/*NN'*-methylenebisacrylamide ratio 50:1, w/w) for monomers to pentamers in the presence of sodium dodecyl sulphate (Laemmli, 1970). A mixed Tris/glycine buffer was employed. Samples (15–20μg) of IgG oligomers were applied to the gels. After development of the gel, protein bands were located by staining with Coomassie Blue.

Determination of the degree of protein modification after cross-linking

Samples of pure oligomers were treated for 2h at 20°C with 2mM-dithioerythritol/150mM-Tris/HCl buffer, pH7.4, followed by treatment with 10mM-iodo[1-^{14}C]acetamide (specific radioactivity 3053c.p.m./nmol). Known quantities of reduced and alkylated samples were loaded on 10% acrylamide gels, and the heavy chains and light chains were resolved by electrophoresis (see above) in the presence of sodium dodecyl sulphate. Bands were located by staining with Coomassie Blue, excised and destained. Sections of the gels were then treated with 1ml of NCS tissue solubilizer (Amersham/Searle, Des Plaines, IL, U.S.A.) at 50°C for 2h. Then 9ml of Bray's (1960) solution was added and the radioactivities of the samples were counted in a Packard Tri-Carb model 3380 liquid-scintillation counter; samples of iodoacetamide used to determine the specific radioactivity of the label were measured in scintillation solution of the same composition as that for the protein samples, including a portion of polyacrylamide gel. The introduction of label corresponding to the alkylation of reduced antibody disulphide bonds was determined with non-cross-linked IgG, and appropriate values were deducted from the observed values for cross-linked material. The results are stated as the average number of carboxamidomethyl residues incorporated per IgG molecule within an oligomer and correspond numerically to twice the average number of cross-linkers incorporated per molecule of IgG in a given complex.

Ultracentrifugation

Sedimentation-velocity and sedimentation-equilibrium studies were performed in a Beckman model E analytical ultracentrifuge at 20°C. The buffer used throughout was 10mM-Tris/HCl buffer, pH7.4, containing 150mM-NaCl. Sedimentation-velocity runs were conducted at 20°C. The sedimentation behaviour was correlated with the assumption of definite geometric arrangements of the IgG within the purified oligomers. Translational frictional coefficients were derived from the theories of Kirkwood (1954) and Bloomfield *et al.* (1967). For these calculation values, values of 1mPa/s (0.01P) for the viscosity (20°C), 0.9982g/cm^3 for the solution density (water, 20°C) and 0.73cm^3/g for the partial specific volume of IgG were used. The immunoglobulin molecule was treated either as the equivalent hydrodynamic sphere of radius 5.7nm or a cylinder of this radius and height. Values of the weight-average molecular weight ($\bar{M}_w$) were obtained from short-column equilibrium centrifugation (Yphantis, 1960). Absorption optics were employed in sedimentation-velocity and sedimentation-equilibrium experiments.

C.d. spectra

These were obtained with a Cary 60 instrument, rebuilt for c.d. measurements, equipped with a Jobin–Yvon modulator at 20°C; protein samples at a concentration of 0.26mg/ml were dissolved in 150mM-NaCl/10mM-Tris buffer, pH7.4, and the spectra were recorded in a 1mm-path-length cell between 250 and 200nm.

Results

Preliminary experiments had indicated that the bivalent reagent dithiobis(succinimidylpropionate) (Lomant & Fairbanks, 1976) was more effective than dimethylsuberimidate in producing IgG oligomers in the range of dimers to pentamers, even though both reagents attack protein α- and ε-amino groups. The first reagent also has the advantage that this cross-linking reagent is cleavable by mild reduction (Lomant & Fairbanks, 1976). The yield of relatively small oligomers is optimal in 0.1M-sodium borate buffer, pH9.0, rather than in phosphate buffer, pH7–9. The sedimentation-velocity pattern of a sample of IgG cross-linked by method B (non-immune IgG, no hapten) disclosed the presence of a significant portion of material sedimenting more rapidly than monomeric IgG, as expected. Two different approaches were employed in the production of oligomers: a large hapten, a 28-unit oligosaccharide derived from the SIII pneumococcal polysaccharide (Jaton *et al.*, 1976), was allowed to organize the specific antibody into an array that was known to fix complement (Jaton *et*

al., 1976). The molecules within such a complex might then be cross-linked as a consequence of their proximity to one another on the hapten, which could be subsequently dissociated at low pH and removed by dialysis. Multiple cross-linking by dithiobis-(succinimidylpropionate) might maintain this orientation or geometry of the individual immunoglobulins within the complex (method A). In parallel to this procedure, the random cross-linking of non-immune IgG molecules in concentrated solutions was exploited to produce large amounts of IgG oligomers, which were expected to possess random arrangements of immunoglobulins within the complex (method B). This method assumes that there is no preferred mode of association of IgG in concentrated solutions (30–40mg/ml) that would lead to the production of oligomers of particular geometry. The application of these two approaches may permit the simultaneous evaluation of the effects of polymerization of IgG and orientation of IgG within the clusters in the expression on antibody effector functions (see Metzger, 1974, 1978).

Pure oligomers of rabbit IgG were obtained by gel filtration of samples that had been cross-linked by dithiobis(succinimidylpropionate) in the presence of hapten (method A) or by the cross-linking of IgG in concentrated solutions of non-immune rabbit IgG (method B). The purified fractions were examined by analytical sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1). Samples were overloaded in order to detect trace amounts of contamination with higher oligomers. All the various fractions exhibited single bands, thus indicating an absolute degree of purity of each oligomer. Oligomers consisting of the monoclonal anti-(pneumococcal SIII polysaccharide) antibody 45–394 had uniformly larger mobilities than those of oligomers composed of non-immune IgG. Identical behaviour was evidenced by two additional preparations of oligomeric IgG series prepared from non-immune IgG of different rabbits. These results are consistent with a more compact geometry of immunoglobulin molecules within aggregates prepared in the presence of hapten by method A. Alternatively, these observations may have reflected electrophoretic differences ascribable to carbohydrate content or variance in the amino acid sequence within the variable regions of the respective constituent IgG molecules.

These aggregates were further characterized by analytical sedimentation-velocity and sedimentation-equilibrium ultracentrifugation (Table 1). The weight-average molecular weights ($\overline{M}_w$) were found to be near multiples of the molecular weight of IgG, supporting the assignments made for the species observed by electrophoretic analysis (Fig. 1). The sedimentation coefficients of the oligomers prepared by method A (hapten present) and method B

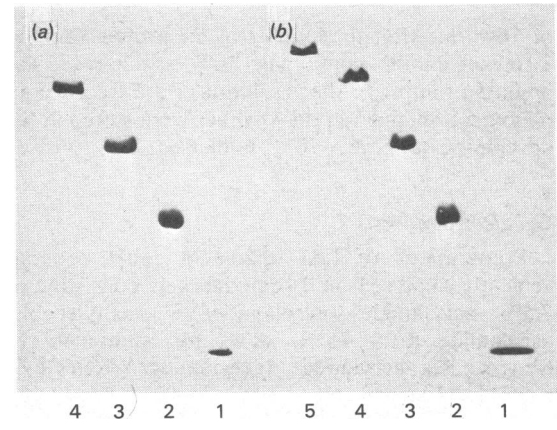

Fig. 1. *Gradient polyacrylamide-gel electrophoresis of purified antibody IgG oligomers (a) and non-immune IgG oligomers (b)*

Samples of the purified oligomers (15–20μg) were analysed on a 3–7.5% polyacrylamide gel in the presence of sodium dodecyl sulphate. The numbers beneath the individual samples correspond to the degree of polymerization of IgG for each sample, calculated from the molecular-weight values obtained in the ultracentrifuge. Both the oligomers obtained by cross-linking in the presence of the multivalent hapten (method A) and the oligomers obtained from the cross-linking of non-immune IgG at high concentration (method B) yielded single bands. The mobility of an oligomer prepared by method B, however, was always slightly less than that of the corresponding oligomer prepared by method A, suggesting perhaps a slightly different arrangement of IgG within the two types of complexes (see the text).

(non-immune rabbit IgG) suggested that the clusters of the same degree of polymerization behave hydrodynamically quite similarly. Observed average sedimentation coefficients of pure dimers, trimers, tetramers and pentamers were 9.7, 11.4, 13.4 and 16.1S respectively (Table 1, column 4). Since the sedimentation coefficient of a complex is related to the translational frictional coefficient, the physical treatments described by Kirkwood (1954) and Bloomfield *et al.* (1967) allowed this coefficient to be computed on the assumption of specific geometries for the oligomers. For doing this, three hydrodynamic models have been considered and are schematically represented in Fig. 2. Oligomers were considered to be cylinders composed of segments of height and radius 5.7nm (Bloomfield *et al.*, 1967) representing the constituent monomers (Fig. 2*a*), or the monomeric elements in the clusters were regarded as spheres hydrodynamically equivalent to single IgG molecules that were arranged in a rigid

Table 1. *Ultracentrifugal characterization of antibody and non-immune IgG oligomers*
The various preparations designated 1A, 2A etc. and 1B, 2B etc. refer to monomers and oligomers prepared by methods A and B respectively (see the Materials and Methods section).

Preparation no.	Assignment	$\bar{M}_w$	Sedimentation coefficient (S) Observed	Theoretical: Bloomfield *et al.*, (1967) Cylindrical	Theoretical: Bloomfield *et al.*, (1967) Linear	Theoretical: Kirkwood (1954) Cyclic
1A		137000	6.5			
	Monomer			—	—	—
1B		—	6.5			
2A		266000	9.8			
	Dimer			9.6	9.6	9.6
2B		281000	9.6			
3A		437000	11.5			
	Trimer			11.6	11.7	12.8
3B		460000	11.2			
4A		—	13.1			
	Tetramer			13.1	13.3	15.0
4B		610000	13.6			
5B	Pentamer	755000	16.1	14.4	14.4	18.1

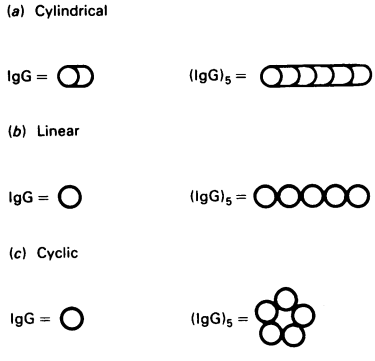

Fig. 2. *Hydrodynamic models for the structure of IgG oligomers*
Model (*a*) is based on the treatment described by Bloomfield *et al.* (1967) and models (*b*) and (*c*) are based on the treatment described by Kirkwood (1954). For details see the text.

linear array (Fig. 2*b*) or in a cyclic array (Fig. 2*c*). The first and second models were based on open extended structures, whereas in contradistinction the third model included a closed arrangement of monomers. The computed values for the sedimentation coefficients of the various oligomers are listed in Table 1 (columns 5–7). The theoretical sedimentation coefficients when the cyclic geometry was considered (Fig. 2*c*) were found to be larger than those for the models based on extended structures (Figs. 2*a* and 2*b*), as anticipated from the compactness of the cyclic structures in contrast with the extended ones. The experimentally determined sedimentation coefficients were in good agreement with those predicted for the cylindrical or linear geometries (Figs. 2*a* amd 2*b*). This correspondence suggested that the aggregates semi-selectively cross-linked in the presence of hapten (method A) and the clusters consisting of IgG cross-linked in random orientations both possessed these open extended geometries.

With the tetrameric and pentameric oligomers prepared by method B, the observed sedimentation coefficients lay somewhat between the theoretical values for linear and cyclic models. This could be a reflection of the lower rigidity of these complexes than assumed in the linear models or of the presence of some isomeric arrangement of the constituent monomers not corresponding to either type of model. Oligomers containing 'branches' would be expected to exhibit sedimentation behaviour intermediate between those of compact and fully extended structures. The lack of agreement of these sedimentation coefficients with the models reflected merely an inadequate hydrodynamic simulation rather than heterogeneity with respect to the number of IgG molecules within the complexes, since these preparations exhibited single bands in analytical sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Moreover, the observed weight-average molecular weights did not hint at a significant degree of heterogeneity, as they were always near multiples of 140000–150000 (Table 1).

To assess the extent of chemical modification of lysine residues in IgG molecules during the preparation of the oligomers, the disulphide bonds in the cross-linking molecules and in the constituent IgG molecules of various oligomers were reduced and alkylated with iodo[1-^{14}C]acetamide, and the heavy chains and light chains of IgG were separated electrophoretically in the presence of sodium dodecyl sulphate. The number of residues modified by the cross-linking agent was then determined (Table 2). As apparent from Table 2, the oligomers produced by method A and method B exhibited a modest degree of modification.

The oligomers prepared by method B lack sufficient cross-links to be cyclic (Table 2), corroborative evidence for the assignment of oligomer geometry on the basis of the sedimentation-velocity analyses. With the oligomers prepared by method A, the degree of cross-linking is somewhat higher. The geometry of these latter oligomers may differ slightly from that of the other series because of the presence of the additional cross-links. This difference may in turn affect the biological properties of these oligomers.

To evaluate the effect of the cross-linking agent on the conformation of the IgG molecules within clusters, c.d. spectra of selected oligomers were taken and compared with that of native monomeric IgG (Fig. 3). The c.d. spectra of dimeric and tetrameric complexes prepared by method B were almost superimposable on the spectrum of untreated IgG from the same serum, exhibiting no shift in the negative maximum at 216nm, with the exception of minor changes in the molar ellipticity at the 212–217nm region and around 230nm. This attested to the essentially unaltered structure of IgG within the oligomers.

The effect of the reduction of cross-linking agent on the sedimentation properties of IgG trimers and pentamers is illustrated in Fig. 4. Reduction converted the trimer and the pentamer into monomeric IgG, as evidenced by the decrease in the sedimentaion coefficients from 11.2 to 6.5S and from 16.1 to 6.4S respectively.

On the basis of several experiments, typical yields of repurified oligomers were 9–20% for dimers, 2–4% for trimers and 1–2% for tetramers. Method A generally exhibited yields at the lower end of these quoted ranges. When the amount of cross-linker was increased from 4 to 6mg/ml, the yield in

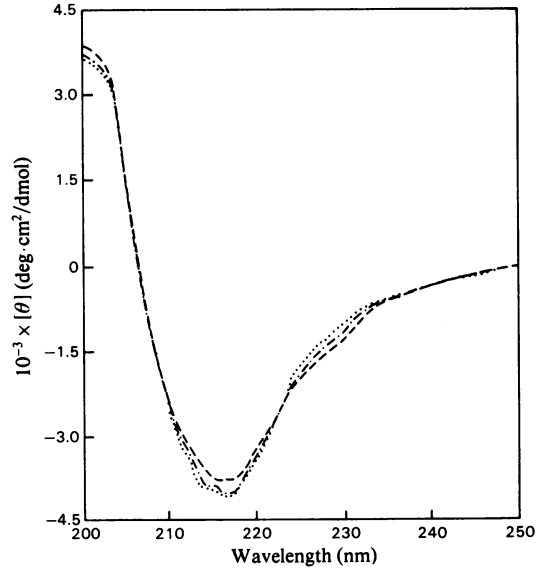

Fig. 3. *Comparison of c.d. spectra of IgG oligomers with that of untreated monomeric rabbit IgG*

The c.d. spectra of untreated rabbit IgG (·····), purified dimer (–·–·–) and purified tetramer (————) were recorded between 250 and 200nm. Within the limits of reproductibility, the spectra do not differ significantly in the wavelength region examined, with the exception of small amplitude differences at 212–217nm and 225–235nm. Therefore the secondary structure of IgG within the oligomers was not grossly altered by the cross-linking procedures. $[\theta]$ is the molar ellipticity calculated from the equation:

$$[\theta] = \theta M / 10cl$$

where θ is the ellipticity measured in degrees, M is the mean residue weight, c is the protein concentration in g/ml and l is the path length of the cell.

Table 2. *Extent of chemical cross-linking in IgG oligomers*

For the values for [1-^{14}C]carboxamidomethyl residues incorporated, corrections were applied for the reduction and alkylation of antibody disulphide bonds (see the Materials and Methods section).

	[1-^{14}C]Carboxamidomethyl residues incorporated (mol/mol)				Cross-linking molecules per oligomer (mol/mol)	
	Method A		Method B			
Species	Heavy chain	Light chain	Heavy chain	Light chain	Method A	Method B
Dimer	0.72	0.78	0.44	0.25	3.0	1.4
Trimer	0.83	0.59	0.30	0.40	4.3	2.1
Tetramer	0.77	0.65	0.44	0.41	5.7	3.4

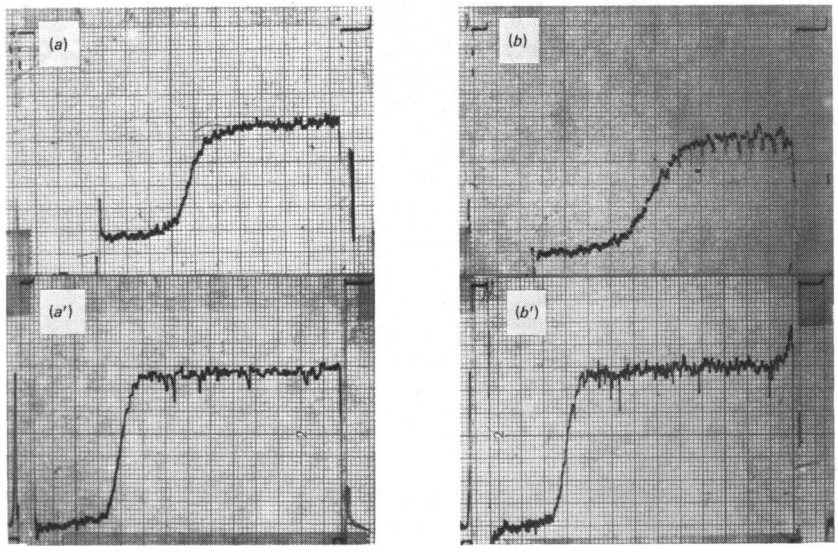

Fig. 4. *Effect of the reduction of cross-linking agent on the sedimentation properties of IgG trimers and pentamers*
Trimeric and pentameric oligomers prepared by method B (no hapten present) were reduced and alkylated as described in the Materials and Methods section. This treatment converted the trimer (*a*) and the pentamer (*b*) into monomeric IgG, as seen in the decrease of the sedimentation coefficients from 11.2 to 6.5 S (*a* and *a'*) and from 16.1 to 6.4 S respectively (*b* and *b'*). The sedimentation profiles were recorded 16 min after the rotor reached a speed of 50000 rev./min.

oligomers was increased, but these more extensively cross-linked molecules lost the ability to bind complement subcomponent C1q (see the accompanying paper, Wright *et al.*, 1980). In order to obtain biological active oligomers, the concentration of cross-linker never exceeded 4 mg/ml. The oligomers were stable for at least 2 months when stored at −70°C, exhibiting no change in sedimentation or electrophoretic properties. In addition, similar experiments indicated that normal human IgG as well as a human IgG_3 myeloma protein gave similar products when oligomers were prepared by method B.

Discussion

To explore the role played by aggregation of monomeric antibody molecule in the elicitation of effector functions mediated by the Fc part of IgG, we have prepared chemically stable aggregates of rabbit immunoglobulin G and purified discrete oligomers to homogeneity. They were obtained by polymerization of IgG monomers by the use of a bifunctional cross-linking agents, dithiobis(succinimidylproprionate). They are stable as long as no reducing agents are present (compare Fig. 1 with Fig. 4). To correlate rigorously antibody-mediated functions such as, for example, fixation or activation of complement with the degree of polymerization of antibody monomers, the antibody oligomers must be stable under physiological conditions over a wide concentration range, exhibiting no tendency to aggregate or dissociate. Previous preparations of IgG oligomers have not completely satisfied this requirement (Hyslop *et al.*, 1970; Segal & Hurwitz, 1976). The oligomers whose preparation is described in detail in the present paper also permit the effect of antibody orientation within the complex on interaction with the first complement component to be assessed. In the first case (method A) antibody was organized into complexes known to fix complement by the addition of a multivalent hapten (Jaton *et al.*, 1976). Through multiple cross-linking this arrangement could be applied in all cases where the antigen or hapten in soluble immune complexes would not be attacked by the cross-linking reagent, thus permitting the isolation of antibody complexes free of antigen or hapten. The danger of re-association of complexes via hapten or antigen is thereby eliminated. Alternatively, non-immune IgG could be cross-linked in concentrated solution. Provided that there is no preferred mode of association of antibody under these conditions, random distribution of Fab–Fab, Fc–Fab and Fc–Fc cross-links are expected. Both the low degree of cross-linking (Table 2) and the lack of significant changes in the c.d.

spectra in the region measured permit us to surmise that the structure of the IgG molecules in the oligomers is essentially the same as that in monomeric untreated IgG (Fig. 3).

Stable defined oligomers of IgG serving as models of antigen–antibody complexes have many applications. The minimal size of an oligomer necessary to activate complement component C1 or C4 may be unambiguously determined. Data in the literature based on the use of unstable cyclic (Hyslop *et al.*, 1970) or undefined (Jaton *et al.*, 1976) complexes suggested that a tetramer or pentamer was the minimum size of an aggregate necessary for complement fixation. Even IgG oligomers, cross-linked in the V-regions by an affinity label, are unstable except when hapten has bound to the antibody (Segal & Hurwitz, 1976).

Stable immunoglobulin E oligomers have been prepared, and investigation indicated that a dimer is the smallest species required to trigger an effector function, degranulation of mastocytes (Segal *et al.*, 1977) in that case. Thus it is of major interest to determine whether dimers of IgG are functional in activating the complement system. These oligomers can be used in electron-microscopic, spectral and kinetic studies of the interaction of IgG with complement subcomponent C1q and component C1. Additionally, neutron-scattering experiments appear to confirm the 'bouquet' model of the complement subcomponent C1q macromolecule (R. A. Dwek, personal communication). The use of the oligomers described in the present paper could be very profitable in assessing the effects of antibody binding on the structure of complement subcomponent C1q. Full details of the use of these oligomers in testing effector functions of antibodies are reported in the accompanying paper (Wright *et al.*, 1980).

J. K. W. gratefully acknowledges the tenure of a scientific assistantship in the Department of Biophysical Chemistry, Biocenter, University of Basel, Basel, Switzerland. The authors thank Ms. E. Steffen for technical assistance and Mr. A. Lustig for ultracentrifugal analysis. This work was supported in part by grant no. 3.146.77 (to J.-C. J.) from the Swiss National Science Foundation.

References

Bloomfield, V., Dalton, W. O. & Van Holde, K. E. (1967) *Biopolymers* **5**, 149–158

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–284

Dudich, E. I., Nezlin, R. S. & Franěk, F. (1978) *FEBS Lett.* **89**, 89–92

Eichman, K. (1972) *Eur. J. Immunol.* **2**, 301–309

Eichman, K. & Kindt, J. J. (1971) *J. Exp. Med.* **134**, 532–552

Goers, J. W., Ziccardi, R. J., Schumaker, V. N. & Glovsky, M. M. (1977) *J. Immunol.* **118**, 2182–2191

Hyslop, N. E., Jr., Dourmashkin, R. R., Green, N. M. & Porter, R. R. (1970) *J. Exp. Med.* **131**, 783–802

Jaton, J.-C., Huser, H., Riesen, F. W., Schlessinger, J. & Givol, D. (1976) *J. Immunol.* **116**, 1363–1366

Kirkwood, J. G. (1954) *J. Polym. Sci.* **12**, 1–7

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Lomant, A. J. & Fairbanks, G. (1976) *J. Mol. Biol.* **104**, 243–261

Metzger, H. (1974) *Adv. Immunol.* **18**, 164–207

Metzger, H. (1978) *Contemp. Top. Mol. Immunol.* **7**, 119–152

Nisonoff, A., Hopper, J. E. & Spring, S. B. (1975) *The Antibody Molecule*, p. 324, Academic Press, New York

Segal, D. M. & Hurwitz, E. (1976) *Biochemistry* **15**, 5253–5258

Segal, D. M., Taurog, J. D. & Metzger, H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2993–2997

Segal, D. M., Guyer, R. L. & Poltz, P. H. (1979) *Biochemistry* **18**, 1830–1838

Willan, K. J., Wallace, K. H., Jaton, J.-C. & Dwek, R. A. (1977*a*) *Biochem. J.* **161**, 205–211

Willan, K. J., Golding, B., Givol, D. & Dwek, R. A. (1977*b*) *FEBS Lett.* **80**, 133–136

Wright, C., Willan, K. J., Sjödahl, J., Burton, D. R. & Dwek, R. A. (1977) *Biochem. J.* **167**, 661–668

Wright, J. K. (1978) *Biochem. Biophys. Res. Commun.* **83**, 1284–1290

Wright, J. K., Engel, J. & Jaton, J.-C. (1978*a*) *Eur. J. Immunol.* **8**, 309–314

Wright, J. K., Engel, J., Brandt, D. Ch. & Jaton, J.-C. (1978*b*) *FEBS Lett.* **90**, 79–83

Wright, J. K., Tschopp, J., Jaton, J.-C. & Engel, J. (1980) *Biochem. J.* **187**, 775–780

Yphantis, D. A. (1960) *Ann. N.Y. Acad. Sci.* **88**, 586–601